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Development of a Respiratory Virus Panel Test for Detection of Twenty Human Respiratory Viruses by Use of Multiplex PCR and a Fluid Microbead-Based Assay▽

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Virology laboratories historically have used direct fluorescent-antibody assay (DFA) and culture to detect six or seven respiratory viruses. Following the discovery of five new human respiratory viruses since 2000, there is an increasing need for diagnostic tests to detect these emerging viruses. We have developed a new test that can detect 20 different respiratory virus types/subtypes in a single 5-h test. The assay employs multiplex PCR using 14 virus-specific primer pairs, followed by a multiplexed target-specific primer extension (TSPE) reaction using 21 primers for specific respiratory virus types and subtypes. TSPE products were sorted and identified by using a fluid microsphere-based array (Universal Array; TmBioscience Corporation, Toronto, Canada) and the Luminex x-MAP system. The assay detected influenza A and B viruses; influenza A virus subtypes H1, H3, and H5 (including subtype H5N1 of the Asian lineage); parainfluenza virus types 1, 2, 3, and 4; respiratory syncytial virus types A and B; adenovirus; metapneumovirus; rhinovirus; enterovirus; and coronaviruses OC43, 229E, severe acute respiratory syndrome coronavirus, NL63, and HKU1. In a prospective evaluation using 294 nasopharyngeal swab specimens, DFA/culture detected 119 positives and the respiratory virus panel (RVP) test detected 112 positives, for a sensitivity of 97%. The RVP test detected an additional 61 positive specimens that either were not detected by DFA/culture or were positive for viruses not tested for by DFA/culture. After resolution of discordant results by using a second unique PCR assay and by using a combined reference standard of positivity, the RVP test detected 180 of 183 true positives, for a sensitivity of 98.5%, whereas DFA and culture detected only 126 of 183 true positives, for a sensitivity of 68.8%. The RVP test should improve the capabilities of hospital and public health laboratories for diagnosing viral respiratory tract infections and should assist public health agencies in identifying etiologic agents in respiratory tract infection outbreaks.

For diagnosing viral respiratory tract infections, clinical virology laboratories historically have used traditional methods such as direct fluorescent-antibody assay (DFA) and culture for the detection of six or seven conventional respiratory viruses. DFA offers a rapid turnaround time for results but is labor-intensive, is subjective, requires trained technologists, and requires specific monoclonal antibodies. With traditional methods, such as DFA and culture, that use microscopy, turnaround times for results are slow, especially in laboratories handling large volumes of respiratory specimens. These methods also are limited by the availability of monoclonal antibodies for newly discovered viruses.

Over the past 10 years, nucleic acid amplification tests have been developed for a number of respiratory viruses. Nucleic acid amplification tests, including PCR and nucleic acid sequence-based amplification, have shown greater sensitivity than DFA and culture (4). Multiplex PCR assays have been used to detect the presence of one or more respiratory virus infections in respiratory tract specimens (1, 3, 5, 6, 8, 9, 10). The emergence of five new respiratory viruses since 2000, including metapneumovirus (MPV), severe acute respiratory syndrome coronavirus (SARS-CoV), avian influenza virus H5N1, CoVs NL63 and HKU1, and human bocavirus, has presented challenges for the virology laboratory. The absence of commercially available tests often leaves laboratories without the ability to diagnose infections with these important emerging viruses. There is, therefore, a need for new and improved diagnostic tests to diagnose both traditional and emerging respiratory virus infections with improved sensitivity. We have developed a multiplex PCR assay, called the respiratory virus panel (RVP) test, that can detect 20 different respiratory viruses, including the orphaned common cold viruses, namely, rhinoviruses and CoVs, not tested for in most clinical laboratories, seven conventional respiratory viruses detected by most clinical laboratories, and emerging viruses, such as MPV, CoVs SARS-CoV, NL63, and HKU1, and avian influenza virus H5N1, that are not detected in routine clinical laboratories (7). The RVP test was more sensitive than DFA and culture and detected 43% additional respiratory virus infections not detected by conventional methods used in the clinical virology laboratory.

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MATERIALS AND METHODS

Specimens. Two hundred ninety-four nasopharyngeal (NP) swab specimens were collected from hospitalized patients in Hamilton, Ontario, Canada, during the winter of 2005 to 2006 with the approval of the Ethics Review Board (St.

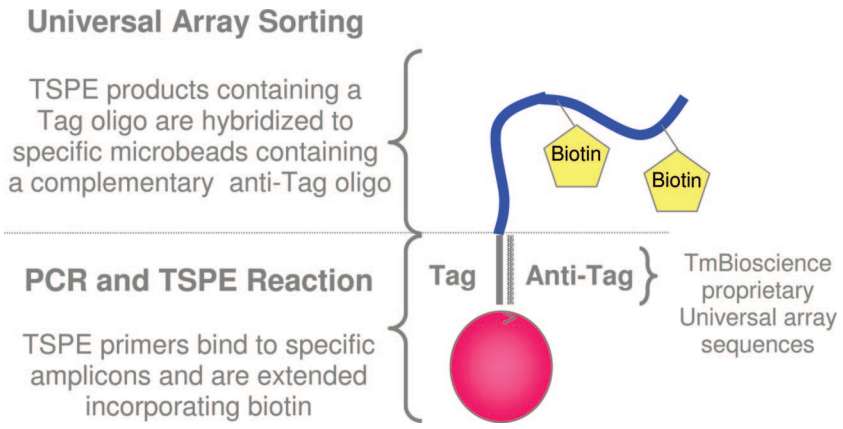

FIG. 1. Detection and identification of TSPE reaction products captured onto microsphere beads containing anti-tag oligonucleotides (oligo) that hybridize to TSPE products containing a complementary tag oligonucleotide. The microbeads are sorted with the Luminex 100 flow cell instrument, which identifies spectrophotometrically colored beads with one laser and a phycoerythrin signal on the beads with a second laser.

Joseph's Healthcare). Consecutive specimens (in 2 to 3 ml universal transport medium; Copan) were collected prospectively and were divided into aliquots. One aliquot (1 ml) was processed in the routine virology laboratory for DFA and shell vial culture, and a second aliquot (0.5 ml) was processed for testing by the multiplex RVP test. DFA was performed using standard methods, and the slides were stained using virus-specific monoclonal antibodies (Diagnostic Hybrids, Inc.) and were read by experienced virology technologists. DFA-negative specimens were set up in shell vial cultures and stained with a panel of eight monoclonal antibodies for 48 h. Shell vial cultures containing R-Mix cells were purchased from DHI.

Nucleic acid extraction. Total nucleic acid (DNA plus RNA) was extracted from aliquots (0.5 ml) of respiratory tract specimens using the Biomerieux MiniMag extractor according to the manufacturer's instructions. Purified nucleic acid (40 μl) was frozen at −80°C in 5-μl aliquots.

RT-PCR. For the RVP assay, a two-step reverse transcriptase PCR (RT-PCR) was used. cDNA was synthesized using Moloney murine leukemia virus RT (Invitrogen) in a 20-μl reaction mixture containing 0.5 μM random hexamers, 0.5 μM deoxynucleoside triphosphates (dNTPs), 1× RT buffer, 0.01 M dithiothreitol, and 5 μl nucleic acid for 60 min at 37°C. cDNA (5 μl) was amplified in a multiplex PCR using 14 primer pairs designed to amplify highly conserved regions of individual viral genomes for detection of specific respiratory virus types and subtypes. The multiplex PCR targeted the following viral genes: the matrix gene of influenza A virus; the three hemagglutinin genes of influenza A virus subtypes H1, H3, and H5; the prehemagglutinin gene of influenza B virus; the hemagglutinin gene of parainfluenza virus types 1 through 3; the phosphoprotein gene of parainfluenza virus type 4; the polymerase gene of respiratory syncytial virus (RSV) types A and B; the hexon gene of adenovirus; the 5′ untranslated region of enterovirus/rhinovirus; the nucleoprotein gene of MPV; and the polymerase gene of CoV. The primers were chosen carefully for target specificity and size so that amplicons would be small (100 to 400 bp) to maximize the amplification efficiency; the median amplicon size was 204 bp. PCR mixtures contained 20 μl PCR master mix, consisting of 1.5× TaKara buffer, 0.15 mM dNTPs, 1 U TaKara *Taq* polymerase, 14 pairs of primers at optimized concentrations ranging from 0.2 to 1 μM, and 5 μl cDNA. The PCR cycling conditions were the following: 1 cycle of 2 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C; and 1 cycle of 2 min at 72°C. Following PCR, the remaining dNTPs and primers were removed by incubating the entire 25-μl PCR mixture with 2.5 U shrimp alkaline phosphatase and 10 U exonuclease (Invitrogen) for 30 min at 37°C, followed by 30 s at 99°C.

TSPE. A multiplex target-specific primer extension (TSPE) reaction was used to detect specific viral sequences amplified by RT-PCR. Treated PCR products (5 μl) were reacted with a mixture of 21 oligonucleotide primers designed to recognize 19 respiratory virus types and subtypes. TSPE primers were chimeric by design, containing both a virus-specific oligonucleotide sequence and a tag oligonucleotide that hybridizes to a complementary anti-tag oligonucleotide bound to 21 spectrofluorometrically labeled microspheres (Fig. 1). The tag and anti-tag oligonucleotides are proprietary sequences and are part of the Universal Array from TmBioscience Corporation (Toronto, Canada). These sequences are unique 3-base, 24-mer oligonucleotide pairs with isothermal hybridization kinetics (2). For the TSPE reaction, an aliquot (5 μl) of the treated PCR product was added to 15 μl of TSPE master mix, containing QIAGEN PCR buffer, dATP, biotinylated dCTP, dGTP, dTTP, 2.5 U TaKara hot-start polymerase, and TSPE primer mix (2.5 μM each). The reaction mixture was incubated with the following cycling conditions: 1 cycle for 2 min at 96°C and then 35 cycles of 30 s at 54°C and 30 s at 72°C.

Analysis of TSPE products using a fluidic microarray. Biotinylated TSPE products were hybridized to a fluid microbead-based array (part of the Universal Array) in wells of a 96-well microtiter plate and were detected using a streptavidin-phycoerythrin conjugate (Molecular Probes, Eugene, OR) (2). The RVP test microbead mix consisted of 21 microbeads, each containing a different fluorescent dye mix and each containing a unique anti-tag oligonucleotide sequence complementary to the oligonucleotide tag sequences incorporated into the 21 TSPE primers. An aliquot of the TSPE reaction mixture (3.5 μl) was mixed with 20 μl of the microbead mix and was incubated for 2 min at 96°C, followed by incubation for 30 min at 37°C. Aliquots (0.1 ml) of streptavidin-phycoerythrin conjugate diluted 1:100 in 1× wash buffer were added to the wells, and the plates were incubated in the dark for 20 min at room temperature. The washed plates were read with a Luminex 100 flow cell instrument after 20 min of incubation. TSPE products bound to specific beads were detected by two lasers in the Luminex 100 flow cell instrument, the first being a red laser used to identify individual microbeads based on unique spectrophotometric dyes incorporated into the microbeads and the second being a green laser used to detect phycoerythrin fluorescence bound to each microbead. The signal on each bead is recorded as the mean fluorescence intensity (MFI) and the output, determined by TDAS software (TmBioscience Corporation), of the instrument that analyzes the raw data and makes a positive or negative determination for each virus type and subtype.

Confirmatory PCR. Twenty confirmatory RT-PCR assays were developed by using a unique pair of primers for each of the 20 virus types/subtypes to confirm positives detected by the RVP assay. Primers were designed in consensus regions of the genome, and optimal conditions for amplification were determined for each set of primers. All amplification targets except for one were chosen outside of the RVP amplicon. For one virus, one primer was located within the amplicon and the other primer was located upstream of it. For the two-step RT-PCR assays, cDNA was synthesized as described above for the RVP assay using random hexamers, and PCR was performed under optimal conditions (primer concentration and annealing temperature) derived for each set of primers. The analytical sensitivity for each confirmatory test was similar to the analytical sensitivity for the corresponding target in the RVP test, as determined by end point titrations using serial dilutions of transcripts (Invitrogen kit) generated in vitro with cloned amplicons.

RESULTS

We designed a multiplex PCR test for the detection and identification of 20 different human respiratory virus types and subtypes, including conventional respiratory viruses, common

TABLE 1. Assignment of virus types and subtypes to individual microbeads for detection by the Luminex x-MAP system

Virus	Bead no.[a]
Influenza A	1
Influenza A subtype H1	2
Influenza A subtype H3	3
Influenza A subtype H5	4
Influenza B	5
RSV type A	6 and 7
RSV type B	6 and 8
MPV	9
Rhinovirus	10
Enterovirus	11
Parainfluenza type 1	12
Parainfluenza type 2	13
Parainfluenza type 3	14
Parainfluenza type 4	15
SARS-CoV	16
CoV 229E	17
CoV OC43	18
CoV NL63	19
CoV HKU1	20
Adenovirus	21

[a] Bead 6 is a common RSV bead detecting both RSV type A and RSV type B. Bead 7 identifies RSV type A, and bead 8 identifies RSV type B.

cold viruses, and newly emerging respiratory viruses. Following nucleic acid extraction, the first step of the assay is a multiplex RT-PCR using 14 consensus primers that amplify consensus regions of the various viral genomes. All PCRs gave specific and robust products, with predominant bands for most viral targets being amplified and only the occasional minor nonspecific band. The second step of the RVP assay involved detection of specific amplicons by using a multiplex TSPE reaction, in which the TSPE primers annealed to specific amplicons and were extended by tag polymerase incorporating biotinylated dCTP. The TSPE reaction used chimeric primers containing a virus-specific sequence and a unique tag sequence that allowed each product to be addressed to a specific microbead that contained a complementary anti-tag oligonucleotide (Fig. 1). TSPE products captured on microbeads were detected with a streptavidin-phycoerythrin conjugate, and signals produced for each bead were analyzed by the Luminex 100 flow cell instrument and expressed as MFI units. A total of 21 microbeads were used in the assay, each capturing a specific PCR product (Table 1). The use of consensus PCR primers together with type- and subtype-specific TSPE primers allowed the detection of the following viruses: influenza A and B viruses; influenza A virus subtypes H1, H3, and H5, including the H5N1 Asian lineage; parainfluenza virus types 1, 2, 3, and 4; RSV types A and B; MPV; adenovirus; rhinovirus; enterovirus; and CoVs OC43, 229E, NL63, HKU1, and SARS-CoV.

The background signals for all 21 beads were low, generally in the range of 50 to 100 MFI units, whereas positive signals were in the 2,000- to 8,000-MFI-unit range, giving signal-to-cutoff ratios in the range of 100 to 500 for all targets (Fig. 2). The low background level on all of the remaining beads (excluding the positive bead) indicates the absence of cross-talk or signal on other beads, indicating the high specificity of hybridization of the Universal Array tag and anti-tag oligonucleotides used for signal sorting. The excellent specificity afforded by the PCR and TSPE primers, together with the excellent signal-to-cutoff ratios, allowed the detection of multiple targets. This detection is shown in Fig. 2 for an influenza A virus, subtype H1N1, that has a strong signal on both the matrix gene bead and the influenza A virus, subtype H1, hemagglutinin gene bead. The high specificity of the assay allowed for the detection of 15 specimens that were positive for two viruses (data not shown).

The analytical sensitivity for each viral target was determined by testing serial dilutions of stock virus seeds (50% tissue culture infectious doses [$TCID_{50}$]/ml) or in vitro-generated RNA transcripts from plasmids containing cloned amplicons (genome equivalents). For all 20 virus types and subtypes

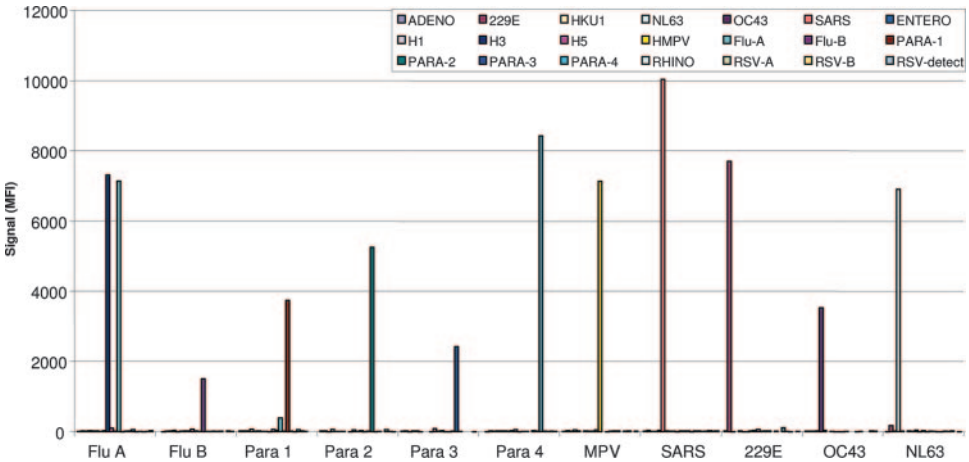

FIG. 2. RVP test results obtained for 11 NP specimens showing TSPE signals recorded for all microbeads. The 11 positive specimens are indicated across the bottom, with the signals for each of the 21 beads indicated by colored bars. The first sample on the left shows a positive signal for the influenza A virus bead (matrix gene positive) and for the influenza A virus subtype H1 hemagglutinin gene. All of the other beads for this sample were negative. ADENO, adenovirus; H1, H3, and H5, influenza A virus, subtypes H1, H3, and H5, respectively; PARA-1 to PARA-4, parainfluenza virus types 1 to 4, respectively; 229E, HKU1, NL63, and OC43, CoVs 229E, HKU1, NL63, and OC43, respectively; SARS, SARS-CoV; Flu-A and Flu-B, influenza A and B viruses, respectively; RHINO, rhinovirus; ENTERO, enterovirus; RSV-A and RSV-B, RSV types A and B, respectively; HMPV, human MPV; RSV-detect, bead 6 (a common RSV bead detecting both RSV type A and RSV type B).

TABLE 2. Distribution of DFA/culture results and RVP test results for 294 NP specimens

No. of specimens	DFA/culture result	RVP test result
123	Positive	Positive
105	Negative	Negative
5	Positive	Negative
14	Negative	Positive
47	Not tested	Positive

tested, the RVP assay detected between 0.1 and 100 $TCID_{50}$ of virus. The RVP test had the following analytical sensitivities: 0.1 $TCID_{50}$ for rhinovirus, enterovirus, CoV 229E, and influenza A virus subtypes H1 and H3; 0.5 $TCID_{50}$ for influenza B virus, parainfluenza virus type 3, and MPV; 1 $TCID_{50}$ for RSV type A and parainfluenza virus type 4; 10 $TCID_{50}$ for parainfluenza virus type 2, RSV type B, and CoVs NL63 and OC43; and 100 $TCID_{50}$ for adenovirus, parainfluenza virus type 1, and SARS-CoV. The corresponding analytical sensitivities in genome equivalents were 50 to 250 for all virus types/subtypes.

We evaluated the performance of the RVP assay by testing 294 respiratory tract specimens that were submitted to the clinical virology laboratory for routine investigation of respiratory viruses. Aliquots of each specimen were tested by routine DFA plus culture, followed by the RVP test. DFA and culture were performed in the clinical virology laboratory, and the RVP test was performed in the research laboratory by technologists blinded to previous results obtained for the specimens. For the 294 specimens, there were 228 concordant results, including 123 positives by DFA/culture and the RVP test and 105 negatives by both tests (Table 2). DFA/culture detected 128 positive specimens, and the RVP test detected 123 of these, for an unadjusted sensitivity of 96.1% for the seven conventional respiratory viruses (influenza A and B viruses, parainfluenza virus types 1 to 3, RSV, and adenovirus) routinely detected in most clinical laboratories. The RVP test detected an additional 61 positive specimens, 14 of which were negative by DFA/culture for the seven viruses tested, and 47 were positive for viruses not tested for by DFA/culture. These 61 additional positive specimens included 2 for influenza A virus, 1 for parainfluenza virus type 1, 2 for parainfluenza virus type 2, 1 for parainfluenza virus type 4, 2 for RSV, 8 for MPV, 39 for rhinovirus/enterovirus, 6 for OC43 CoV, 2 for NL63 CoV, 1 for HKU1 CoV, and 3 specimens that were positive for two viruses, including 1 specimen that was positive for MPV and rhinovirus/enterovirus and 2 specimens that were positive for OC43 and rhinovirus/enterovirus. All of the 66 specimens that gave discordant results, including the 5 DFA/culture-positive specimens that were negative by the RVP test and the 61 specimens that were positive by the RVP test and negative or positive for viruses not tested for by DFA/culture, were tested by a second PCR that targeted a different area of the viral genome. Table 3 shows the results for the 5 specimens that had given DFA/culture-positive, RVP test-negative discordant results and for the 14 specimens that had given DFA/culture-negative, RVP test-positive discordant results. Three of the 5 DFA/culture-positive, RVP test-negative specimens (numbers 167, 191, and 187) were confirmed to be positive by PCR,

TABLE 3. PCR results for 5 DFA/culture-positive, RVP test-negative and 14 DFA/culture-negative, RVP test-positive specimens

Specimen no.	Result by test[a]:		
	DFA/culture	RVP (MFI reading)	Second PCR[b]
167	Flu B^+	Flu B^- (42)	Flu B^+
191	Para 2^+	Para 2^- (13)	Para 2^+
286	Para 2^+	Para 2^- (29.5)	Para 2^-
62	Para 1^+	Para 1^- (20)	Para 1^-
187	RSV^+	RSV^- (93)	RSV^+
108	Flu A^-	Flu A^+ (298)	Flu A^+
53	Para 2^-	Para 2^+ (266)	Para 2^+
349	Para 2^-	Para 2^+ (6447)	Para 2^+
89	Para 1^-	Para 1^+ (215)	Para 1^+
443	RSV^-	RSV^+ (213)	RSV^-
58	Flu A^-	Flu A^+ (412)	Flu A^-
128	Mpn^-	Mpn^+ (7229)	Mpn^+
441	Mpn^-	Mpn^+ (1837)	Mpn^+
503	Mpn^-	Mpn^+ (286)	Mpn^+
549	Mpn^-	Mpn^+ (656)	Mpn^+
566	Mpn^-	Mpn^+ (966)	Mpn^+
601	Mpn^-	Mpn^+ (6032)	Mpn^+
604	Mpn^-	Mpn^+ (3537)	Mpn^+
119	Mpn^-	Mpn^+ (6772)	Mpn^+

[a] Flu A, influenza A virus; Flu B, influenza B virus; Para 1, parainfluenza virus type 1; Para 2, parainfluenza virus type 2; Mpn, metapneumovirus.
[b] A second confirmatory PCR targeting a unique genomic region was performed to resolve the discordant results, as described in Materials and Methods.

indicating two false positives (numbers 286 and 62) by DFA/culture. All of the additional 61 RVP test-positive specimens were confirmed as true positives by the second PCR. If a true positive is defined as being positive by two or more tests (DFA, culture, the RVP test, and/or confirmatory PCR), then there were 183 positives and 111 negatives. To determine how the RVP test performed compared to DFA and culture, we eliminated the 47 specimens that were positive for a virus not tested for by DFA and culture (i.e., parainfluenza 4; MPV; CoVs OC43, 229E, NL63, and HKU1; and rhinovirus/enterovirus) and used the remaining 247 specimens for analysis. Among these 247 specimens, there were 137 positives and 110 negatives. The sensitivity and specificity of DFA/culture were 91.9% (126/137) and 98.2% (108/110), respectively. The RVP test had a sensitivity of 97.8% (134/137) and a specificity of 96.4% (107/110). If, however, all confirmed respiratory viruses detected by the RVP test are included in the analysis, then the RVP assay detected 180 out of 183 positive specimens and had an overall sensitivity of 98.4%, whereas DFA/culture detected only 126 out of 183 specimens and had a sensitivity of 68.8%.

Of particular interest was the finding that 15 out of 294 (5.2%) specimens were positive for two viruses in this group of specimens. The dual infections included the following combinations: one type 1 parainfluenza virus plus one rhinovirus/enterovirus, one type 2 parainfluenza virus plus one rhinovirus/enterovirus, two type 3 parainfluenza viruses plus one rhinovirus/enterovirus, three RSVs plus one rhinovirus/enterovirus, one adenovirus plus one rhinovirus/enterovirus, one MPV plus one OC43 CoV, three MPVs plus one rhinovirus/enterovirus, two OC43 CoVs plus one rhinovirus/enterovirus, and one adenovirus plus one KHU1 CoV. No specimen was positive for three respiratory viruses. Testing additional spec-

imens from a separate study has confirmed a dual positivity rate for NP specimens collected from our combined pediatric and adult population of 5% to 8% using the RVP assay during the 2005-to-2006 season.

DISCUSSION

The RVP test detects 20 respiratory viruses, including the conventional respiratory viruses influenza A and B viruses, parainfluenza virus types 1 to 4, RSV, adenovirus, MPV, common cold viruses such as rhinovirus, CoVs OC43 and 229E, and newly emerging respiratory viruses, such as SARS-CoV, avian influenza virus H5N1, and CoVs NL63 and HKU1, which usually are not tested for by clinical laboratories. The assay employs a multiplex PCR using 14 primer pairs, followed by a multiplex TSPE reaction using 21 primer pairs, to detect and identify 20 different virus types and subtypes in a single test. The test uses a 96-well microtiter plate format and the Luminex 100 flow cell instrument. Since the current test involves several steps and is unable to produce results as quickly as DFA, a shortened version of the test currently under development will cut the run time significantly and allow faster turnaround times for results. Following nucleic acid extraction, the RVP assay takes about 5 h to perform, making it possible for some laboratories to provide same-day results. The actual turnaround time for results will vary from laboratory to laboratory, depending on specimen volumes. One technologist can easily handle two plates and can generate results for up to 190 specimens plus controls in one work shift, recognizing that the extraction may be the rate-limiting factor, not the RVP test itself. Laboratories that have two automated extractors would be able to process 192 specimens per day. If routine PCR contamination precautions are taken, the risk of false positives can be minimized and should be no higher than that obtained with any PCR test. We have not seen PCR contamination issues with the RVP assay in testing over 2,000 specimens, since both the PCR and the TSPE reaction are performed in closed 8-well capped strips and only the hybridization step is performed in open wells of 96-well plates. For this reason, we have not used uracil-*N*-glycosylase in the PCR master mix. For the investigational-use-only and subsequent in-vitro-diagnostic-device versions of the assay, the manufacturer has added two controls to the test. For the first control, an aliquot of MS2 RNA phage is added to each specimen prior to nucleic acid extraction. This controls for nucleic acid extraction and also serves as an amplification inhibitor control. The second control is lambda phage DNA, which is included in every run as a run control to control for amplification and detection.

The analytical sensitivity of the RVP test for the 20 different viruses ranged from 0.1 to 1 $TCID_{50}$, corresponding to approximately 50 to 250 genome equivalents. The differences in analytical sensitivities obtained for genome equivalents and $TCID_{50}$ determinations likely reflect the production of viral interfering particles in cell culture and/or the presence of viral transcripts.

To determine the clinical performance of the RVP assay, we compared the results of the RVP assay to those of DFA/culture by using 294 prospectively collective specimens submitted to the regional virology laboratory for investigation of respiratory viruses. Excluding the 47 specimens that were positive for a virus not tested for by DFA and culture (i.e., parainfluenza virus type 4; MPV; CoVs OC43, 229E, NL63, and HKU1; and enterovirus/rhinovirus), the sensitivity of DFA/culture was 92% (126/137), while the sensitivity of the RVP test was 97.8% (134/137). The major advantage of the RVP test is the ability to identify an additional eight respiratory viruses not detected by DFA/culture, including five CoVs, parainfluenza virus type 4, rhinovirus, and enterovirus, and to identify influenza virus subtypes H1, H3, and H5 and RSV types A and B. In this study, the RVP test detected 42% more viruses than DFA/culture (180 detections by the RVP test compared to 126 detections by DFA/culture). All of the additional positives were confirmed as true positives by a second PCR assay that used different primers. The overall sensitivity for detecting any respiratory virus was 98.5% (180/183) for the RVP test and 68.8% (126/183) for DFA/culture. The specificity of the RVP test (96.4%) was similar to that of DFA/culture (98.2%). The sensitivities of the RVP assay for detecting individual viruses was 83% (5/6) for influenza B virus, 92% (11/12) for parainfluenza virus type 2, 96.6% (28/29) for RSV, 100% (10/10) for influenza A virus, 100% (19/19) for parainfluenza virus type 1, 100% (17/17) for adenovirus, 100% (22/22) for MPV, and 100% (10/10) for parainfluenza virus type 3. The numbers of positives for some viruses were quite low, however, and the true sensitivity of the RVP assay for individual viruses must await larger studies.

In resolving the RVP test-positive, DFA/culture-negative results for some specimens, only a single confirmatory PCR was performed to confirm that the additional positives were true positives and not false positives. If these specimens yielding discordant results were tested by all 21 confirmatory PCR tests, some additional viruses may have been detected, and this may have changed the performance characteristics of the RVP assay, as well as those of DFA/culture. The RVP test provides a much broader coverage for respiratory viruses than DFA and culture, which detect only seven or eight viruses. Other multiplex PCR assays have been described that detect up to 7 viruses in a single test or up to 14 viruses in two multiplex reactions (3). The specificity of the RVP assay and its ability to detect multiple viruses in a single reaction is achieved by the aggregate specificity of primers and oligonucleotides used for the PCR, the TSPE reaction, and the Universal Array hybridization steps. A commercial version of the RVP test, called ID-Tag RVP, presently is in clinical trials. This version will include an internal control that is spiked into the specimen to control for extraction of nucleic acid and a run control. The broad coverage of the RVP test should improve the ability of clinical laboratories to diagnose respiratory virus infections in hospitalized patients and assist public health laboratories in identifying viral etiological agents in respiratory tract infection outbreaks in the community, where laboratories typically detect as few as 25% to 30% of infections by use of traditional methods (M. Smieja, C. McNally, M. Loeb, M. Fearon, L. Burton, A. Haley, B. Suggett, M. Taha, S. Richardson, et al., presented at the International Conference on Antimicrobials and Chemotherapy, Washington, DC, 30 October to 2 November 2004). The RVP test should increase our understanding of the epidemiology of respiratory viral infections. Much of our knowledge of respiratory virus epidemiology comes from studies performed in the 1970s, when laboratory tests were limited

and insensitive. Use of the RVP test in broad population-based studies will increase our knowledge of the seasonality and risk factors for respiratory virus infections in different patient populations. For example, the RVP test indicated that there were up to 12 respiratory viruses cocirculating in Hamilton in the months of January and February in 2006, whereas DFA and culture detected five viruses that cocirculated in the community the previous winter. Using the RVP assay, we have consistently seen a dual respiratory virus infection rate of 5% to 8% for symptomatic patients and even some triple virus infections. In the current study of 294 NP specimens, we found positives for RSV and influenza A virus, RSV and parainfluenza virus type 3, and RSV and MPV, combinations that have been reported in the literature, plus new combinations of viruses not previously reported, including influenza A virus with MPV, parainfluenza virus type 3 with rhinovirus/enterovirus, MPV with rhinovirus/enterovirus, and MPV with CoV OC43. It is not known if infections with multiple respiratory viruses are associated with adverse outcomes or increased hospital stays in specific patient populations. Clinical studies are ongoing to determine whether dual respiratory virus infections carry an increased risk for adverse outcomes or increased hospital stays for pediatric and adult patients.

The RVP test also may be useful in the global surveillance of emerging or reemerging respiratory viruses, such as SARS-CoV or the avian influenza virus H5N1. The RVP test was designed to detect all influenza A virus subtypes but not to identify specific H or N types. An RVP test signal on the influenza A virus matrix gene bead in the absence of a signal on the type H1, H3, or H5 bead would be consistent with a new influenza type and would indicate the need for further genotyping of the isolate. The reemergence of a new influenza A virus, such as H7N2 or H9N2, into the human population would be detected by the RVP test, which detects the matrix gene of all influenza A virus genotypes H1 through H16 (data not shown). The RVP test has detected H1, H3, H5, H7, and H9 (data not shown) and therefore could act as a sentinel test for the global surveillance of newly emerging influenza viruses. Since the assay detects the currently circulating avian influenza virus H5N1, the assay also can be used to signal the migration of H5N1 into new areas of the world.

REFERENCES

1. **Bellau-Pujol, S., A. Vabret, L. Legrand, J. Dina, S. Gouarin, J. Petitjean-Lecherbonnier, B. Pozzetto, C. Ginevra, and F. Freymuth.** 2005. Development of three multiplex RT-PCR assays for the detection of 12 respiratory RNA viruses. J. Virol. Methods **126:**53–63.
2. **Bortolin, S., et al.** 2004. Analytical validation of the Tag-It high-throughput microsphere-based universal array genotyping platform: application to the multiplex determination of a panel of *Thrombophilia*-associated single-nucleotide polymorphisms. Clin. Chem. **50:**2028–2036.
3. **Coiras, M. T., J. C. Aguilar, M. L. Garcia, I. Casas, and P. Perez-Brena.** 2004. Simultaneous detection of fourteen respiratory viruses in clinical specimens by two multiplex reverse transcription nested-PCR assays. J. Med. Virol. **72:**484–495.
4. **Falsey, A. R., M. C. Criddle, and E. E. Walsh.** 2006. Detection of respiratory syncytial virus and human metapneumovirus by reverse transcription polymerase chain reaction in adults with and without respiratory illness. J. Clin. Virol. **35:**46–50.
5. **Fan, J., K. J. Henrickson, and L. L. Savatski.** 1998. Rapid simultaneous diagnosis of infections with respiratory syncytial viruses A and B, influenza viruses A and B, and human parainfluenza virus types 1, 2, and 3 by multiplex quantitative reverse transcription-polymerase chain reaction-enzyme hybridization assay (Hexaplex). Clin. Infect. Dis. **26:**1397–1402.
6. **Kaye, M., S. Skidmore, H. Osman, M. Weinbren, and R. Warren.** 2006. Surveillance of respiratory virus infections in adult hospital admissions using rapid methods. Epidemiol. Infect. **134:**792–798.
7. **Mahony, J. B., S. Chong, F. Merante, K. Luinstra, T. Sinha, A. Petrich, C. Lisle, S. Yaghoubian, and R. Janeczko.** 2006. Development of a multiplex nucleic acid amplification assay for the detection and identification of 17 human respiratory viruses, abstr. C-284. Abstr. 106th Ann. Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, DC.
8. **Osiowy, C.** 1998. Direct detection of respiratory syncytial virus, parainfluenza virus, and adenovirus in clinical respiratory specimens by a multiplex reverse transcription-PCR assay. J. Clin. Microbiol. **36:**3149–3154.
9. **Scheltinga, S. A., K. E. Templeton, M. F. Beersma, and E. C. Claas.** 2005. Diagnosis of human metapneumovirus and rhinovirus in patients with respiratory tract infections by an internally controlled multiplex real-time RNA PCR. J. Clin. Virol. **33:**306–311.
10. **Templeton, K. E., S. A. Scheltinga, M. F. Beersma, A. C. Kroes, and E. C. Claas.** 2004. Rapid and sensitive method using multiplex real-time PCR for diagnosis of infections by influenza A and influenza B viruses, respiratory syncytial virus, and parainfluenza viruses 1, 2, 3, and 4. J. Clin. Microbiol. **42:**1564–1569.